



# Children with growth hormone (GH) deficiency treated with recombinant human GH have an increased frequency of CD57<sup>+</sup> T and CD56<sup>+</sup> NK cells and an augmented IFN- $\gamma$ production in lymphocytes

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## Abstract

**Background:** The administration of recombinant human growth hormone (rhGH) has been reported to restore the immune function due to GH deficiency (GHD). We herein investigated the phenotypic characterization, interferon (IFN)- $\gamma$  production ability, and apoptosis of peripheral blood mononuclear cells (PBMCs) from patients with idiopathic GHD treated with rhGH to elucidate the effect of rhGH therapy on Th1 immune responses.

**Methods:** PBMCs were obtained from either 14 GHD patients treated with rhGH, 22 healthy children or five healthy adult volunteers. The lymphocyte subsets were analyzed by a flow cytometric analyzer and cultured with anti-CD3 antibody, interleukin (IL)-2 and IL-12, or staphylococcal enterotoxin B, and thereafter the IFN- $\gamma$  levels were evaluated.

**Results:** The proportions of CD57<sup>+</sup> T cells and CD56<sup>+</sup> NK cells increased more in the patients than in healthy children. The IFN- $\gamma$  production in the lymphocytes from the patients was also greater than that from healthy children. The CD3-stimulated lymphocytes from the patients were less susceptible to apoptosis. When the lymphocytes from healthy adults were cultured with anti-CD3 antibody and various concentrations of rhGH, the IFN- $\gamma$  levels in the culture supernatants increased in a dose-dependent manner.

**Conclusions:** These findings suggest that rhGH therapy can enhance the IFN- $\gamma$  production of lymphocytes probably due to a proportional increase in the number of CD57<sup>+</sup> T cells and CD56<sup>+</sup> NK cells as well as due to the inhibition of lymphocyte apoptosis.

**Keywords:** growth hormone, CD57<sup>+</sup> T cell, CD56<sup>+</sup> NK cell, IFN- $\gamma$ , apoptosis

## Background

Growth hormone (GH) is a neuroendocrine hormone produced by the somatotroph cells of the anterior pituitary [1]. GH is considered important for the regulation, function, and normal development of the immune system [2]. The GH receptor is a member of the hematopoietin/cytokine receptor family [3] and is expressed on human peripheral blood mononuclear cells (PBMCs) [4]. Recently, GH was reported to regulate the immune system, and GH deficiency (GHD) can cause impairment in proinflammatory cytokine production [5]. GH administered extrinsically acts as a lymphopoietic, thymopoietic and T-cell stimulating factor under certain circumstances [3]. In addition, the administration of recombinant human GH (rhGH) has been reported to restore immune function that is lowered due to GHD [5-9]. However, high doses of rhGH therapy have been shown to be associated with increased morbidity and mortality in aged patients with prolonged critical illnesses [10]. Although the precise mechanism regarding

the effects of rhGH is unclear [10], rhGH therapy has been suggested to have either a beneficial or an adverse effect on the immune system in humans *in vivo* [11,12].

$\alpha\beta$ T cells with natural killer (NK) cell markers such as CD56 or CD57 are present in the peripheral blood (PB) of humans [13,14]. These NK-T cells produce a greater amount of interferon (IFN)- $\gamma$ , which is well known as a representative T helper (Th) 1 cytokine [15]. CD57<sup>+</sup> T cells (most of which are CD8<sup>+</sup>) in PB increase proportionally with age, chronic infectious diseases, and oncological diseases [15-17]. They have a very limited T cell receptor repertoire, thus suggesting that they develop in a thymus-independent manner [13,18]. In addition, CD57<sup>+</sup> T cell-expansion has been suggested to play a role in the development of acute graft-versus-host disease [19]. Therefore, NK-T cells together with NK cells could be crucial effectors in the host immune system, especially with respect to Th1 immune responses in humans [13,20].

GH reportedly increases the number of NK cells and

NK activity in patients with GHD [4]. It was suggested that GH could effect the differentiation and proliferation of NK cell progenitors or precursors in the bone marrow [21]. In addition, GH enhances developing and peripheral T cell migration [22]. However, the effect of GH on NK-T cells remains to be elucidated.

We herein investigated the phenotypic characterization, IFN- $\gamma$  production ability, and apoptosis of PBMCs from patients with idiopathic GHD treated with rhGH to elucidate the effect of rhGH therapy on Th1 immune responses.

## Subjects and Methods

Fourteen patients (11 boys and 3 girls) diagnosed as having GHD because of their short stature with a peak value of GH in two provocative tests below 10.0 ng/mL [23] were studied. At the time of the present study, they had been treated with rhGH (0.175 mg/kg/week) for at least 1 year with an average age of  $11.7 \pm 0.9$  (mean  $\pm$  S.D.) years and an average serum insulin-like growth factor (IGF)-1 concentration of  $638 \pm 135$  (mean  $\pm$  S.D.) ng/mL, which was higher than in the healthy children (mean value, 267 ng/mL at 11-12 years of age), thus indicating that the patients responded well to the therapy [23]. Twenty-two children (11 boys and 11 girls, with an average age of  $9.7 \pm 0.9$  years) who visited the outpatient clinic of National Defense Medical College Hospital for routine examinations and five healthy adult male volunteers (an average age of  $36.0 \pm 2.6$  years) were also studied. All patients and healthy children were considered to be preadolescent by physical examinations at the time of the present study. In general, there are no significant differences between prepubertal boys and girls concerning about the levels of GH and IGF-1, and the response to rhGH therapy in GHD patients [23]. Heparinized PB samples were obtained from the patients, healthy children, and adult volunteers from the cubital vein. The protocol was reviewed and approved by the ethical committee for human studies of the National Defense Medical College. Informed consent was obtained from the parents of all children.

## Cell isolation, monoclonal antibodies (mAb), and flow cytometric analysis

PBMCs were obtained from the heparinized PB using a Lymphocyte Separation Medium (ICN Biomedicals Inc., Aurora, OH, USA). The lymphocytes maintained a high viability (>90%) during the observation period. The surface phenotypes of PBMCs were identified by mAb in conjunction with three-color immunofluorescence tests. The mAb used in this study were as follows: fluorescein isothiocyanate (FITC)-anti-CD57 Ab (NC1) or FITC-anti-CD3 Ab (UCHT1), phycoerythrin (PE)-anti- $\alpha\beta$  T-cell receptor (TCR) Ab, PE-anti-CD57 Ab or PE-anti-CD56 Ab (NKH-1) and phycoerythrin-cyanin 5.1 (PC5)-anti-CD56 Ab or PC5-anti- $\alpha\beta$  TCR Ab, all of which were purchased from Beckman Coulter (Miami, FL, USA). The lymphocytes were gated by forward scatter

and side scatter and then the surface phenotypes of PBMCs were analyzed by a flow cytometric analyzer (EPICS XL, Beckman Coulter). In this analysis, CD56<sup>+</sup> T cells included CD56<sup>+</sup> CD57<sup>+or-</sup>  $\alpha\beta$ TCR<sup>+</sup> cells [15].

## Cell cultures

PBMCs were adjusted to a concentration of  $1 \times 10^6$  cells/mL with RPMI 1640 containing 20% human serum. One hundred  $\mu$ L (5  $\mu$ g/mL) of anti-CD3 Ab (UCHT1, PharMingen, San Diego CA, USA) had been incubated overnight at 4 °C in 96-well flat-bottomed plates to immobilize the Ab, and then the plates were washed three times before starting the culture. Two hundred  $\mu$ L of the adjusted cells were cultured with immobilized anti-CD3 Ab or 100 ng/mL of interleukin (IL)-2 (Pepro Tech EC, London, UK) and 2 ng/mL of human IL-12 (Pepro Tech EC) or 10  $\mu$ g/mL of staphylococcal enterotoxin B (SEB, Sigma, St, Louis, MO, USA) in 96-well flat-bottomed plates in 5% CO<sub>2</sub> at 37 °C. PBMCs from adult volunteers were cultured with immobilized anti-CD3 Ab and increasing concentrations ( $10^{-3}$  to  $10^5$  ng/mL) of rhGH (Humatrope, Lilly, Kobe, Japan) or IL-2 and IL-12 or SEB in the presence of  $10^3$  ng/mL of rhGH in the same manner as that described above. After a 48-hour culture, the supernatants were harvested and stocked at -80 °C for an enzyme-linked immunosorbent assay (ELISA). In some other experiments, the CD3-stimulated cells were harvested and then subjected to an assay for lymphocyte apoptosis after the 48-hour culture.

## Assays for IFN- $\gamma$ levels

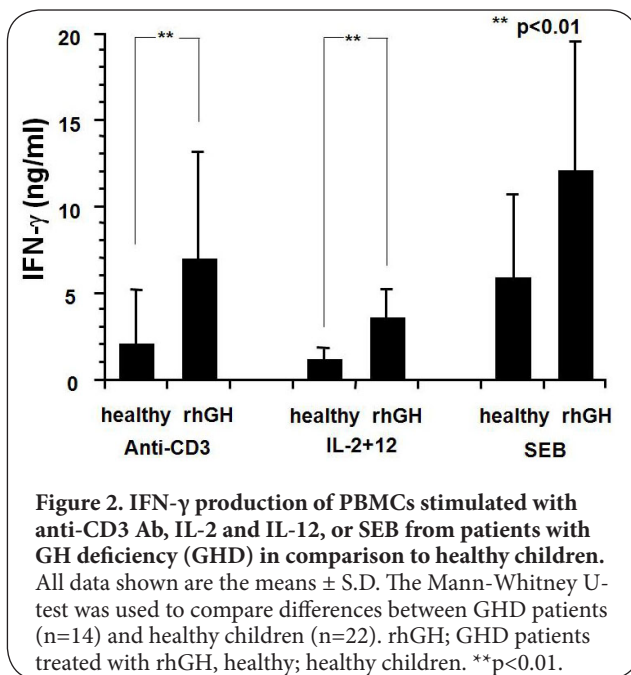
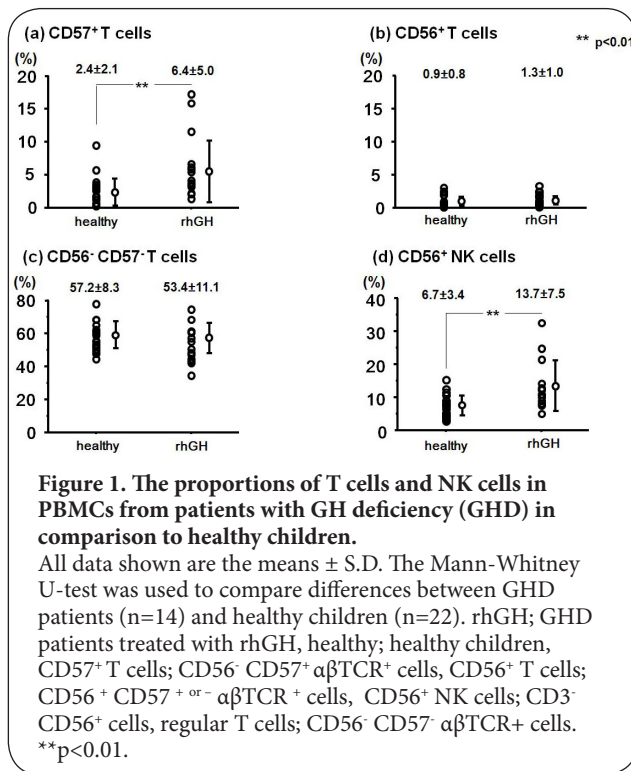
The IFN- $\gamma$  levels in the culture supernatants were assayed using the cytokine-specific ELISA (OptEIA™, PharMingen).

## Assay for lymphocyte apoptosis

An assay for reactivity to annexin V [24] in apoptotic cells was performed using commercial reagents (Immunotech, Marseille, France) according to the manufacturer's instructions. After staining the cells with FITC-annexin V and propidium iodide (PI), the cells were applied to a flow cytometric analyzer (EPICS XL, Beckman Coulter). The apoptotic ratio was calculated as the annexin V-positive and both PI-negative and PI-positive fractions (i.e., early and late apoptotic cells, respectively) in whole lymphocytes [25].

## Statistical analysis

All values were expressed as the means  $\pm$  S.D. The Mann-Whitney U-test was used to compare differences between unpaired samples, while the Wilcoxon signed rank test was used to compare any differences between paired samples. The effect of rhGH on the IFN- $\gamma$  production from PBMCs of adults *in vitro* was evaluated by one-way ANOVA, followed by the Scheffé' F. A statistical analysis was performed using the Statview software package (version 5.0). Differences were considered to be significant when the p value was < 0.05.



## Results

### The proportions of T cells and NK cells in PBMCs from patients

We first analyzed the proportions of T-cell and NK-cell subsets in PBMCs from both patients with GHD treated with rhGH and healthy children. The results showed that the proportions of CD56<sup>-</sup> CD57<sup>+</sup>  $\alpha\beta$ TCR<sup>+</sup> (CD57<sup>+</sup> T) cells and

CD3<sup>-</sup> CD56<sup>+</sup> NK cells in PBMCs were significantly larger in GHD patients treated with rhGH than in healthy children (Figure 1a, d). However, there were no significant differences in the proportions of CD56<sup>+</sup>  $\alpha\beta$ TCR<sup>+</sup> (CD56<sup>+</sup> T) cells and CD56<sup>-</sup> CD57<sup>-</sup>  $\alpha\beta$ TCR<sup>+</sup> (regular T) cells between the groups (Figure 1b, c). Although CD56<sup>+</sup> T cells include CD56<sup>+</sup> CD57<sup>+</sup> T cells, their proportion was less than 0.1% in individuals under 20 years of age [20]. Then, we analyzed the proportions of T-cell and NK-cell subsets in PBMCs from healthy adults. The results showed that the proportions of CD57<sup>+</sup> T cells, CD56<sup>+</sup> T cells, regular T cells, and CD56<sup>+</sup> NK cells were  $8.8 \pm 5.6\%$ ,  $2.7 \pm 0.8\%$ ,  $51.1 \pm 5.2\%$ , and  $15.5 \pm 6.5\%$ , respectively.

### IFN- $\gamma$ production ability of PBMCs from patients after various stimulations

Although the stimulation by anti-CD 3 Ab is not a natural antigen stimulation, this stimulation has been considered to represent T-cell function in antigen-specific T-cell responses (i.e., against viruses) [26]. Th1 cytokines such as IL-2 and IL-12 are known to activate T cells and NK cells to produce a large amount of IFN- $\gamma$  [13]. SEB is considered as bacterial superantigens which are known to bind major histocompatibility complex (MHC) class 2 and broadly activates T cells through their T cell receptors without antigen presenting cells [13].

After stimulation with immobilized anti-CD3 Ab or IL-2 and IL-12 for 48 h, PBMCs from the patients produced larger amounts of IFN- $\gamma$  than those from healthy children (p<0.01, Figure 2). Although PBMCs from the patients stimulated with SEB tended to produce a larger amount of IFN- $\gamma$  than those from healthy children, the difference was not statistically significant.

### Apoptosis of PBMCs from patients in response to CD3-stimulation

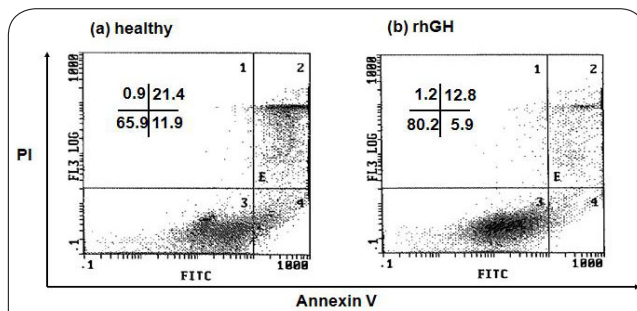
After the stimulation of PBMCs with anti-CD3 Ab for 48 hr, the apoptotic ratio of the stimulated PBMCs from patients showed a lower value than those from healthy children ( $20.5 \pm 4.0$  versus  $29.5 \pm 4.0\%$ , respectively, n=5, p<0.05). Representative results are shown in Figure 3.

### The effect of rhGH on IFN- $\gamma$ production of PBMCs from adults *in vitro*

When PBMCs from adult healthy volunteers were cultured with anti-CD3 Ab in the presence of several concentrations of rhGH, the IFN- $\gamma$  levels in the culture supernatants increased in a dose-dependent manner (Figure 4a). Furthermore, PBMCs from adult volunteers were cultured with IL-2 and IL-12 or SEB in the presence of  $10^3$  ng/mL of rhGH, PBMCs produced larger amounts of IFN- $\gamma$  than those from PBMCs without rhGH (p<0.05, Figure 4b).

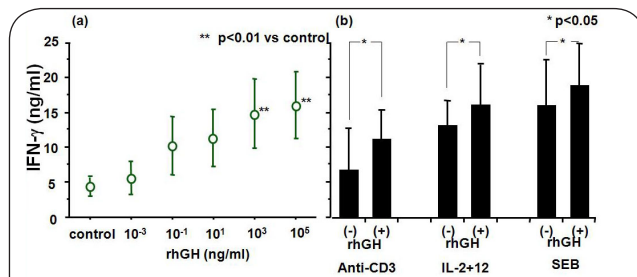
## Discussion

In the present study, we have shown that children with GHD treated with rhGH have an increased frequency of CD57<sup>+</sup> T



**Figure 3. Apoptosis of PBMCs in response to CD3-stimulation from patients with GH deficiency (GHD) in comparison to healthy children.**

PBMCs from a healthy child (a). PBMCs from a patient with GH deficiency treated with rhGH (b). Representative results are shown from repeated experiments with similar results. The apoptotic ratio was calculated as the annexin V-positive and both PI-negative and PI-positive fractions (i.e., early and late apoptotic cells, respectively) in whole lymphocytes.



**Figure 4. The effect of rhGH on IFN- $\gamma$  production of PBMCs from adult healthy volunteers.**

IFN- $\gamma$  production of PBMCs stimulated with anti-CD3 Ab from adult volunteers in the presence of increasing concentrations of rhGH (a). All data shown are the means  $\pm$  S.D. based on PBMCs from five adult volunteers. The effect of rhGH on IFN- $\gamma$  production was evaluated by one-way ANOVA, followed by the Scheffe' F. \*\* $p < 0.01$  vs control (without rhGH). IFN- $\gamma$  production of PBMCs stimulated with anti-CD3 Ab, IL-2 and IL-12, or SEB from adult volunteers in the presence of 103 ng/mL of rhGH (b). All data shown are the means  $\pm$  S.D. based on PBMCs from five adult volunteers. The Wilcoxon signed rank test was used to compare differences between the groups. \* $p < 0.05$  vs control (without rhGH).

we showed that CD57<sup>+</sup> T cells as well as CD56<sup>+</sup> NK cells, but not regular T cells, increased proportionally in the PB of patients treated with rhGH for at least 1 year. Although we did not evaluate the proportions of PBMCs in subjects before initiating the therapy, the results suggest that rhGH treatment induced an increase in the numbers of these cells in the periphery. It was suggested that GH could effect the differentiation and proliferation of NK cell progenitors or precursors in the bone marrow [21]. Therefore, GH may also play an important role in the development of lymphocytes in a thymus-independent manner. This may be one of the possible explanations for the increase in the peripheral T cells bearing CD57 (one of the human NK cell markers) and CD56<sup>+</sup> NK cells increased in patients treated with rhGH.

Another notable finding was that PBMCs from the patients produced a large amount of IFN- $\gamma$  in response to anti-CD3, Th1 cytokines, or bacterial superantigens. Pagani *et al.*, also reported that 3 months of GH treatment ameliorated the production of tumor necrosis factor- $\alpha$  and IFN- $\gamma$  in PBMCs from GHD children [5]. They speculated that both NK cells enhanced by the therapy and macrophages activated by these cytokines play important roles in the proinflammatory cytokine-induced immune response in humans. Based on our previous studies, CD57<sup>+</sup> T cells are activated to produce IFN- $\gamma$  by either anti-CD3 or SEB-induced IL-12, while CD56<sup>+</sup> NK cells are activated by IL-2 and IL-12 to produce IFN- $\gamma$  [15,32]. Furthermore, we previously reported that IFN- $\gamma$  production in a one-way mixed lymphocyte reaction correlated with the proportion of CD57<sup>+</sup> T cells in responder cells [19]. Therefore, the proportional increase of CD57<sup>+</sup> T cells and CD56<sup>+</sup> NK cells in PBMCs is considered to play an important role in the augmentation of IFN- $\gamma$  production. We also showed that rhGH augmented IFN- $\gamma$  production from CD3-stimulated PBMCs of healthy adults in a dose-dependent manner. rhGH may affect the IFN- $\gamma$ -producing ability itself [21,33] because human lymphocytes express GH receptors [2,4]. However, the concentration of 10<sup>3</sup> ng/mL of rhGH *in vitro* used in the present study was extremely high (physiological serum GH levels are usually less than 50 ng/mL) [23]. This phenomenon is partly because GH receptor expression on T and NK cells is lower than that on B cells in healthy adults [34]. Therefore, high-dose rhGH therapy may be needed to produce a clinical effect on the immune system.

GH exerts an anti-apoptotic effect during stress [3]. Our findings also suggest that rhGH treatment can inhibit lymphocyte apoptosis. We recently demonstrated that CD57<sup>+</sup> T cells are more susceptible than regular T cells to apoptosis [15,35]. We speculate that activated CD57<sup>+</sup> T cells may die after inducing Th1 immune responses in the hosts to avoid any additional harmful effect [15,35].

The clinical significance of the relationship between GH and cytokine production in humans is debatable [5]; however, GH has been suggested to be an immunomodulating therapeutic agent [3]. rhGH may be a promising agent for the regulation of the immune system for hematopoietic stem

and CD56<sup>+</sup> NK cells, whose levels are closely similar to those of over 40-year-old healthy adults [15], and augmented IFN- $\gamma$  production in their lymphocytes compared to healthy children. Although patients with GHD do not usually demonstrate an immunocompromised status [27], decreased CD4/CD8 ratios [28] and diminished NK cell activities [29,30] have been reported in these patients. Smaniotto *et al.*, showed that GH might exert an effect on T cell trafficking from the thymus to the PB [31]. Goodier *et al.*, reported that CD3<sup>-</sup> CD56<sup>+</sup> NK cells in PB increased more in patients with HIV infection receiving both rhGH and highly active antiretroviral therapy (HAART) than in those receiving HAART alone [21]. In the present study,

cell transplantation, acquired immunodeficiency syndrome, post chemotherapy, infectious diseases including severe sepsis, and normal aging [36,37]. However, the effects of clinically administered GH remain a debatable matter with respect to immunotherapy [38]. This may be partly because the causes and the specific treatment of the underlying diseases are heterogeneous.

This study had several limitations. First, we encountered difficulties in obtaining adequate blood volumes required for some experiments from children, and therefore could not investigate the exact roles of CD57<sup>+</sup> T cells and CD56<sup>+</sup> NK cells in patients with GHD treated with rhGH. Second, children with GHD not treated with rhGH might be more appropriate as a control group. Finally, the immunological changes in our patients described above seem to not result in any clinical difference in comparison to healthy children. These limitations will be addressed in future research.

## Conclusion

Our findings suggest that rhGH therapy augments the IFN- $\gamma$  production of lymphocytes probably due to a proportional increase in the number of CD57<sup>+</sup> T cells and CD56<sup>+</sup> NK cells as well as due to the inhibition of lymphocyte apoptosis. rhGH therapy may not affect clinical immunological features in children with GHD, but cause a controversial effect under certain circumstances.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

YK carried out the studies, performed the statistical analysis and drafted the manuscript. SS conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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